TOXICODISTRIBUTION OF MERCURY AND SELENIUM

IN PINNIPEDS OF ALASKA

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A

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Abstract

This study is divided into two major parts (chapters) in order to better understand mercury (Hg) and selenium (Se) tissue distribution in pinnipeds. The first part of the study focuses on determining total mercury ([THg]) and selenium ([TSe]) concentrations (mass and molar based) among cardiac and renal tissues of ice seals (focus on bearded seals, *Erignathus barbatus*) as compared to the more traditionally analyzed tissues (e.g. liver, skeletal muscle). Determining Hg distribution within these tissues is essential in establishing sampling methods for biomonitoring, histopathology and biochemistry of Hg. Age was determined to be an important driver of [THg] and Se:Hg molar ratios in heart and kidney. In bearded seals [THg] varied by heart region and therefore future studies should use consistent sampling methods in order to determine and compare [THg]. Despite the differences in seal kidney structure when compared to many terrestrial mammals, the kidney cortex was the main accumulation site for Hg within the kidney of bearded seals and requires consideration in sampling designs. Se:Hg molar ratios greater than 1 in all tissues can be considered a baseline for normal Se concentrations under relatively low [THg].

The second part of the study focuses on THg and TSe distribution in Steller sea lion (*Eumetopias jubatus*) pup tissues in addition to THg tissular and body burdens. Hair had the highest [THg] in all 5 Steller sea lion pups as compared to other tissue compartments. Since these pups were 1-2 months of age, the hair (lanugo) sampled was a good indicator of Hg exposure via maternal placental transfer (in utero) and potentially a good indicator of individual THg tissue burdens. The percent of total Hg body burden for many organs in Steller sea lion pups was similar to that found in Pacific harbor seals. The Se:Hg molar ratios were between 1 and 50 in all tissues of 4 of the 5 pups while the pup with the highest [THg] in all tissues, had Se:Hg molar ratios of 0.7 or less in 9 of 14 tissues indicating that this animal may have limited Se-dependent protection related to Hg toxicosis.

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Dedication

This piece of work is dedicated to
my nephew, Manuel J. Villa and niece, Savanna R. Villa.
Hard work and dedication is the key to success.
The key to happiness is
finding the courage within yourself to do the thing you love the most.
Be curious and explore the world around you.
Never stop asking questions,
you never know what new adventure lies ahead.
Always hope for the best
but be prepared for the worst.
And remember that God is
everywhere in nature
and it is through science
that we begin to understand
the true meaning of his creation.

GENERAL INTRODUCTION

Mercury in the environment

Mercury (Hg) is present within the earth's crust at an average concentration of 50 parts per billion (ppb), however, it may be found localized in natural waters at less than 2 ppb (seawater = 40 part per trillion) and in soils between 0.1-0.5 ppb (as high as 200 ppb in contaminated soils) [1]. Soils are long term reservoirs of Hg and one of the main sources of Hg to freshwater systems [1,2]. Natural mobilization of Hg may occur during volcanic activity, forest fires and geological processes such as weathering of rocks [1–3]. Over the past several hundred years, human activities such as coal burning and mining have produced atmospheric Hg emissions, with increasing emissions over the past few decades [3]. Deposition of Hg from the atmosphere to the environment (freshwater, marine, terrestrial) occurs in either wet or dry forms [3–6]. Wet deposition, also referred to as precipitation, is mostly in the form of rain or snow. Dry deposition is the contamination of the surface without precipitation. More than 90% of Hg in terrestrial environments resides in soils and is largely bound to organic matter and sulfur (S) [6–8].

In the Arctic, Hg has been well studied for the past several years and provides insight into high latitude Hg cycling. The Arctic Ocean receives several tons of Hg per year by inflow from the Atlantic and Pacific Ocean and over a hundred tons through deposition from the atmosphere [3]. In the Arctic Ocean, Hg deposition is dominated by atmospheric deposition of Hg [3,6,8–10]. In addition to atmospheric deposition, recent studies have shown that delivery of Hg to the Arctic Ocean is also via northward flowing rivers such as the Mackenzie River in Canada [5,6] and many Russian rivers [11] . A large portion of river runoff into the Beaufort Sea of the Arctic Ocean comes from the Mackenzie River as well as the Yukon River Basin [3,6,12]. The biogeochemical cycle of Hg in river systems is complex and dependent on local environmental conditions.

The erosion and weathering of sulfur (S)-containing sediments and rocks in freshwater environments is a critical component in the Hg cycle. Carrie et al. [6] found that in mountainous zones of the Mackenzie River Hg was predominately bound to S-containing particles and was released into runoff or sediment discharge during oxidation and weathering of HgS compounds. HgS compounds have strong stability constants (log K ~ 38, [13]) and are not considered biologically available [6,7]. Erosion and weathering of coal contributes about 10% of total Hg inputs to the Mackenzie River and only about 6% of total Hg comes from atmospheric deposition [6]. Approximately 88% of Hg in the Mackenzie River originates from sediments in mountainous zones and about 90% of all the Hg delivered to the Beaufort Sea is in the form of HgS [6]. Similarly, approximately 90% of total Hg in the Yukon River Basin is in particulate form, potentially bound to S. The majority (about 98%) of all total Hg exports from the Yukon River Basin occur during spring and summer. Schuster et al. [12] noted that the Hg yield from Yukon River Basin are greater than those from the Mackenzie River and other northern hemisphere rivers potentially due to increasing terrestrial Hg from permafrost thawing as well as delayed glacial melt periods during the summer. Harris et al. [14] provides an illustration of Hg cycling and bioaccumulation in marine environments.

Various species of Hg can either be oxidized to a higher valence state or reduced to a lower valence state and thus can exist in various forms within the environment. Gaseous elemental mercury (Hg^0) is most abundant in the atmosphere [3,15]. Hg^0 is highly volatile and easily transported through wind currents and eventually re-deposited into the environment where it can be oxidized to divalent mercury (Hg^{2+}) [3,8,10,15]. Hg^{2+} is most abundant in freshwater, marine, and terrestrial environments [3,6,8]. Hg^{2+} can exist in the atmosphere but only for a short time before rain water, snow or adsorptive processes aid in deposition of Hg to the environment [8]. Hg^{2+} is highly soluble in water, reactive and less volatile than Hg^0. Transformation of Hg^{2+} to organic

Hg, monomethylmercury ($MeHg^+$), occurs within marine, freshwater and terrestrial environments. $MeHg^+$ formation occurs more readily in areas with low levels of oxygen such as sediments and wetlands as well as the mid-water column of the Arctic Ocean [3]. Methylation of Hg^{2+} to produce $MeHg^+$ can occur through microbial processes [3].

Mercury toxicology

Toxicity of Hg is dependent on the bioavailability and chemical form of Hg which in turn is dependent on both environmental and physiological factors. The chemical form of Hg will dictate toxicodistribution. Various forms of Hg target organs such as the liver, kidney, brain and heart [15]. Hg^0 is a volatile gas that can have potential impacts on the respiratory and cardiovascular system. Once in the blood stream, Hg^0 can be oxidized to Hg^{2+}. The association of Hg with coronary heart disease, myocardial infarction and hypertension is limited to Hg^0 vapor exposure in humans [16,17] and not enough is known about specific element distribution or concentrations within the pinniped heart. $MeHg^+$ has been known to have adverse effects on reproductive, immunological, and neurological functions [3,18–21]. $MeHg^+$ crosses the blood-brain barrier as well as other membrane structures such as the placenta and gastrointestinal tract [22–24]. $MeHg^+$ is absorbed through the intestinal wall of fish, readily transported by blood throughout the body and primarily accumulates in the muscle [3,25]. $MeHg^+$ distribution is systemic in the sense that it targets vital organs such as the brain and accumulates in several tissues such as erythrocytes in blood, muscle, and hair [21]. On the other hand, Hg^{2+} is found in greater concentrations mostly in two target organs, the liver and kidney. Several studies have found both liver and kidney have demethylating mechanisms that convert $MeHg^+$ to Hg^{2+} [21,26,27]. Certain kidney diseases have been associated with high concentrations of Hg^{2+} accumulation in the cortex and outer medulla of humans and rats [21].

As determined previously, Hg is known to promote oxidative stress in various organs such as the kidney and heart [28–30]. Through Fenton-like reactions, Hg may influence the conversion of oxygen (O_2) to superoxide ($O_2^{\bullet -}$) [30]. Oxidative stress is caused by the formation of excess free radicals within the cell or organelles. Free radicals are highly reactive and can damage lipids, proteins, and nucleic acids [31–33]. In biological systems, oxygen free radicals are also referred to as reactive oxygen species (ROS) which include $O_2^{\bullet -}$ and hydrogen peroxide (H_2O_2). When found in excess, several ROS have been implicated in various diseases including cancers [31]. Under normal conditions, immune cells use ROS to generate bactericidal agents such as hypochlorous acid, hydroxyl radicals and peroxynitrites [34,35]. Natural defenses against the formation of excess ROS in biological organisms involve the activity of the glutathione peroxidase (GPx) enzyme and many other systems.

Antioxidants: Emphasis on Se

As an example, in most systems the main function of GPx is to reduce lipid hydroperoxides and hydrogen peroxides to hydroxyl compounds and water, respectively [32]. Selenium (Se), an essential element, is a key cofactor for the catalyzing mechanism of GPx and the peptide glutathione (GSH) is an important substrate for GPx [33]. This antioxidant reaction helps protect cells from oxidative damage caused by free radicals. An increase or decrease in Se availability will dramatically impact the activity of GPx and its ability to prevent oxidative damage [33]. As an essential element, Se is under homeostatic regulation. Dietary Se is associated with amino acids and is found in plant and animal food sources as well as water. In the blood stream, Se is more commonly in the form of selenocysteine or selenomethionine and interacts with GPx to form selenodiglutathione, elemental Se (Se^{2-}), selenites (SeO_3^{2-}) and hydrogen selenide (HSe^-) [36].

Selenium is under homeostatic control not only due to its importance in enzymatic activity but also likely due to its direct interactions with toxic elements. Post gastrointestinal absorption, interactions between Se and Hg may occur in the blood stream since both elements have a high affinity for the sulfhydryl groups of certain blood proteins and enzymes and are transported into the liver and kidney [37,38]. In the blood stream, SeHg binding is in part driven by the availability of glutathione (GSH). Hg binding to GSH can result in the depletion of GSH in the kidney and the increased production of H_2O_2 [17,38,39] . In a previous study, the presence of Se after the ingestion of Hg prevented GSH depletion in the liver of rats and rabbits [39]. In addition, Se can increase Hg half-life in the blood and liver making it less reactive and have a significant effect in organ distribution and excretion of Hg [38,39].

It has been hypothesized that an abundance of Se as compared to Hg on a molar basis, where Se:Hg molar ratio is well above 1, is important for potential amelioration of the adverse effects due to $MeHg^+$ exposure [3,38,40,41] and possibly Hg^{2+}. This antagonistic relationship between Se and Hg has been studied in various organisms; however, the mechanism(s) for this relationship remains to be elucidated. Several reactions and compounds have been hypothesized to be important in this relationship. While HgS bonding occurs spontaneously, HgSe bonds are favored because they are stronger, require a greater formation constant and result in a more stable compound [38,39]. The formation of HgSe complexes is dependent on the reduction of Se by GSH to HSe^-. Analytical results demonstrated that in the blood, a HgSe compound is bound at the core of a GSH containing compound [39]. This (GSH)HgSe complex will then bind to selenoproteins and decrease Hg bioavailability [38]. (GSH)HgSe complex is also the major transporter of Hg into the liver and kidney.

The presence of Hg-Se granules in the liver and kidney of some marine mammals has provided evidence for the mechanisms underlying the analytical chemical observations of highly correlated Hg and Se concentrations in the liver

and kidney [38,42]. In laboratory animals exposed to Hg^{2+} and SeO_3^{2-} simultaneously, granules formed in the liver and kidney. These are most likely HgSe compounds bound to selenoprotein P forming a complex which can be further transformed into a crystalline HgSe complex [38,43]. However, since biological systems are high in sulfides, the granules observed in marine mammals are likely to be inert, mineralized products of a HgSeS complex generated after demethylation of $MeHg^+$ [38]. The insoluble HgSe complex, also referred to as the mineral tiemannite, can be generated by a direct reaction between Hg and HSe, through the degradation of (MeHg)Se or through the dissociation of HgSe bound to selenoproteins [38,44,45]. Khan and Wang [38] provides an illustration of a few possible HgSe complexes that may result in a decrease of Hg bioavailability and thus aid against $MeHg^+$ toxicosis.

Mercury and piscivorous marine mammals

Fish consumption is the main route of exposure of $MeHg^+$ for piscivorous marine mammals [3,46]. Biomagnification of Hg in part is the increase in $MeHg^+$ concentration from prey to predator. Bioaccumulation of $MeHg^+$ can be defined as an increase of $MeHg^+$ concentration over the life span of an organism made possible by consistent dietary intake and slow elimination rates. Both biomagnification and bioaccumulation result in higher levels of $MeHg^+$ in top predators such as piscivorous marine mammals compared to lower trophic level organisms. As top predators, piscivorous marine mammals can serve as models or sentinels of $MeHg^+$ exposure for other species [47,48]. Similar to Hg, Se is also introduced through the diet and biomagnifies resulting in higher Se concentrations in marine mammals when compared to terrestrial mammals. Due to the role of Se as an antioxidant and its direct interactions with Hg it is essential to assess Se distribution in target organs as well as various tissues of the body. Diving mammals such as seals and sea lions are at a greater risk of lipid peroxidation than nondiving mammals due to extended periods of ischemia

(restriction of blood flow) and reperfusion (returning to a normal state) during foraging. Episodes of ischemia and reperfusion in diving mammals generate an excess amount of $O_2^{\bullet -}$ in organs such as the kidney, requiring greater antioxidant activity [49].

Bearded seals are a subsistence food source for the human populations of the western and northern coasts of Alaska and across the Arctic. Bearded seals have been studied previously and are known to feed at lower trophic levels than most seals and sea lions [50,51]. The distribution of bearded seals in Alaska extends from the Beaufort Sea to the Chukchi and Bering Seas [52]. The preferred habitat for bearded seals is packed sea ice further offshore than other ice seals such as ringed and spotted seals. The Alaska Department of Fish and Game in cooperation with the Alaska Native subsistence harvests began a biomonitoring program in 1962 in order to evaluate population health and status of bearded seals as well as other ice seals essential to Alaska Natives [51,53]. Several parameters are measured in order to determine changes in health and population status of bearded seals. These parameters include growth rate, body condition, diet, age distribution and pregnancy rate among others. In addition, tissue samples are collected and analyzed for diseases, and contaminants such as organochlorines (OC) and heavy metals.

The toxic effects of Hg are well known for humans and terrestrial mammals but not for marine mammals such as ice seals, providing the need for background and reference values of Hg concentrations in healthy seals. Both Hg and Se concentrations tend to be higher in marine mammals than in terrestrial mammal, due to biomagnification of both Hg and Se, which is a potential indication of the antagonist relationship between Hg and Se as seen in other species [38,41,43]. Bearded seals are an important component to this project because they provide a unique opportunity to analyze freshly collected and quickly frozen tissues of target organs (i.e. kidney and heart) from an ice seal species. Further subsampling of the heart and kidney into specific regions (e.g.,

renal cortex and renal medulla) is important in improving histopatholgical and biochemical sampling methods for seals and sea lions when evaluating Hg toxicosis as well as proper sampling for biomonitoring programs.

Steller sea lion populations in Alaska are genetically and geographically divided into distinct population segments (DPS), the western and the eastern. The eastern DPS extends from southeast Alaska to California. The western DPS extends from approximately Cape Suckling, Alaska (144^{o} W) in the Gulf of Alaska to the western extent of the Aleutian Islands. Both DPS experienced population declines during the 1970s and 1980s. Since then the eastern DPS, particularly in southeast Alaska and southern Oregon, has increased slightly and is currently listed as a threatened species [54–56]. The dramatic and continued decline of the western DPS is still under study and is the cause for their status as endangered species. Several causes for the western DPS decline have been proposed such as nutritional stress, fisheries competition and pollution among others but conclusive evidence has not been shown [55,57–59]. Subsistence hunting as well as illegal hunting has been excluded as significant threats. In recent years, pollution which includes exposure to contaminants such as polychlorinated biphenyls (PCB) and heavy metals (e.g., Hg) has been under study as a potential cause for the lack of recovery of Steller sea lions in the western DPS [55,60–62].

The Alaska Department of Fish and Game in cooperation with National Marine Fisheries Service is currently monitoring Steller sea lion contaminant exposure and assessing maternal diet through sampling of blood, hair and whiskers of pups between the ages of 1-2 months for heavy metal and carbon and nitrogen stable isotope analysis. Since it is well known that Hg can affect reproductive success and pup survival, blood and hair are analyzed for Hg exposure. Recently, Hg concentrations in blood and hair of Steller sea lion pups have been found to be higher in the western Aleutian Islands when compared to southeast Alaska [60–62] and in some cases have exceeded the

human and wildlife threshold for Hg adverse effects. Steller sea lion pups that were opportunistically collected when found freshly dead on breeding rookeries present a unique opportunity to determine Hg distribution and Hg burden in the body of sea lion pups at both relatively low and high Hg concentrations.

The Steller sea lion pups in this study were found dead at various islands and do not represent a random sample from the population. However, data on the distribution of THg (mass and molar concentrations) among different tissues (compartments) of these pups enable wildlife agencies to better interpret the concentrations of THg measured in the less invasive hair samples collected from a random sample of the free ranging population of Steller sea lion pups across the region.

Thesis objectives

The objectives of this research study were to:

1. Determine and compare Hg and Se concentrations (mass and molar based) within and between cardiac tissue (by chamber) and renal (cortex and medulla) tissue compartment of bearded seals and other ice seals (Chapter 1),
2. Compare Se:Hg molar ratio among cardiac tissue and renal tissues of bearded seals (Chapter 1),
3. Determine Hg concentrations and Hg burdens in various tissue compartments and the total body burden of Hg of Steller sea lions (Chapter 2), and
4. Compare Se:Hg molar ratios in Steller sea lion tissues (Chapter 2).

Chapter 1 examines the Hg and Se concentrations of specific regions of the heart and kidney (chambers, cortex, and medulla) of free-ranging bearded seals and other ice seals that were collected during subsistence hunts. Knowledge of Hg distribution within the heart and kidney can help determine

improved sampling methods for biomonitoring, histopathology and biochemistry (oxidative and antioxidant mechanisms). In addition, we calculate the ratio (molar concentrations) of Se to Hg in order to better understand the potential defense mechanism against Hg toxicosis in marine mammals. The heart regions analyzed include samples of the left and right ventricles, left and right atria and the interventricular septum. The kidney regions analyzed include the cortex and medulla. Since Hg and Se concentrations within these tissues have not been well documented, skeletal muscle and liver will be used as reference tissues for the heart and kidney, respectively since Hg and Se concentrations for these tissues are well known in the literature and will be used as reference points.

Chapter 2 evaluates Hg distribution (compartment based Hg concentration and burden) in sea lion pup carcasses collected from the wild and its associated compartment burden (e.g., total mass of specific element in the liver), and body burden (total mass of element in the entire organism or sum of all compartments). Hg tissue burden is important in understanding the full extent of Hg toxicodynamics. We compare Hg concentrations from all tissues obtained, taking into account mass of tissue, to determine which tissue has the highest Hg concentration and burden. We also evaluate the Se:Hg molar ratio in all tissues. Se:Hg molar ratio varies among tissues of the body and can provide insight on the potential Se-based defense mechanisms.

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CHAPTER 1:

Mercury and selenium in heart, kidney, skeletal muscle and liver of ice seals from Alaska: Focus on bearded seals[1]

1.1 Abstract

Total mercury (THg) and selenium (TSe) concentrations (mass and molar based) were determined in different heart and kidney regions of bearded seals as compared to more traditionally analyzed tissues (e.g. liver, skeletal muscle). Determining THg concentration ([THg]) within and between these tissues is important to our understanding of how Hg can affect biological functions of known target organs and their use in biomonitoring programs. Se:Hg molar ratios are important in determining potential elemental associations and possible antioxidant protection against Hg toxicosis. In the current study, age was determined to be an important driver of [THg] and SeHg molar ratios in heart and kidney. Comparison of mean [THg] in five heart regions revealed significant differences among the four chamber walls and the interventricular septum ($p < 0.05$). Mean [THg] ranking in bearded seals was: liver > kidney cortex > kidney medulla > skeletal muscle > heart left ventricle ($p < 0.001$). Mean [TSe] were greater in kidney cortex and kidney medulla than in the heart left ventricle. Mean Se:Hg molar ratios were ranked: heart left ventricle > kidney medulla > kidney cortex. Se:Hg molar ratios were significantly greater than 1.0 in all tissues ($p < 0.001$) and can be considered a baseline for normal Se concentrations under relatively low Hg concentrations as compared to other tissues.

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Keywords: mercury, selenium, cardiac tissue, renal tissue, molar ratio

1.2 Introduction

The toxic effects of mercury (Hg) are well known for humans and terrestrial mammals but not well characterized for marine mammals. In some species of marine mammals, especially older piscivorous animals, the liver has higher Hg concentrations ([THg]) than the kidney making the liver a traditional tissue of study for "worst case scenarios" [1,2]. Skeletal muscle has been studied to assess Hg exposure in marine mammals and as a subsistence food. Skeletal muscle is considered a major storage site of monomethylmercury ($MeHg^+$), a toxic form of Hg that bioaccumulates and biomagnifies [3].

Selenium (Se) is an essential trace element with concentrations tending to be higher in some marine mammals than in terrestrial mammals, and in many studies [THg] and Se concentrations ([TSe]) have been shown to be positively correlated [4,5]. One of the earliest studies regarding Hg-Se interactions in marine mammals was conducted by Koeman et al [6] who found a high correlation between [THg] and [TSe] in liver and kidney and suggested that a Se:Hg molar ratio greater than 1 could be beneficial by forming HgSe compounds and detoxifying $MeHg^+$ [5]. In addition, Se can act directly as an antioxidant or as a component of key antioxidants such as glutathione peroxidase (GP_x). The activity of GP_x has been used as an indicator of antioxidant defenses and Se status within various tissues of the body [7,8]. Enzymes such as GP_x are essential in preventing cellular damage under oxidative conditions which can lead to lipid peroxidation in some organs. Diving mammals such as bearded seals are at a greater risk of lipid peroxidation than nondiving mammals due to extended periods of ischemia (restriction of blood flow) during diving and reperfusion once returning to the surface [7,9]. In addition to diving, Hg intake can serve as an additional oxidative stress. Due to the potential antioxidant role of Se and its direct interactions with Hg, it is also

important to understand the distribution of both [THg] and [TSe] in organ tissues such as heart and kidney.

The distribution of [THg] in heart and kidney tissues of marine mammals has not been well documented and less is known about Hg adverse effects in marine mammal species in general. Hg distribution is potentially different within the different regions of the heart and kidney due to the varying degrees of vascularization and concentration of sulfur-containing compounds. Some sulfur-containing compounds include glutathione (GSH) and myoglobin which are known to bind to various forms of Hg [10–13]. The heart is morphologically similar across mammalian species. The kidney morphology, however, varies across mammalian species with some species such as seals having discrete multireniculated structures with many cortical and short medullary structures compared to non-reniculated kidneys [14,15].

The three species of seals studied here are used for subsistence in western and northern Alaska (bearded seals, *Erignathus barbatus*: ringed seals, *Pusa hispida*, and spotted seals, *Phoca largha*). Mercury (Hg) and other potentially toxic contaminants are a concern for Native peoples that rely on seals for food, therefore a better understanding of [THg], distribution, and whether the presence of Se has potential protective effects are important for seals and humans. In the heart and kidney, Hg can have interactive effects resulting in hypertension or heart disease. In addition, a deficiency or toxicosis of Se in the heart and kidney can potentially alter their function. In this study we evaluate [THg] in heart (for each chamber wall and the septum) and in kidney (cortex and medulla) and compare these concentrations with the more commonly studied liver and muscle [16–18] in order to understand the distribution of [THg] within and among organs. We evaluated [TSe] in heart and kidney to calculate and compare Se:Hg molar ratios in the heart to Se:Hg molar ratios in the kidney, as well as to unity (Se:Hg = 1) in order to assess the relative potential defense mechanisms against Hg toxicosis.

1.3 Materials & Methods

1.3.1 Sample collection

Heart (n=50), kidney (n=49), muscle (n=50), and liver (n=51) samples from ice seals (ages: <1 – 30 years old) were provided by the Alaska Department of Fish and Game (ADF&G) in cooperation with the Alaska Native subsistence harvest (ADF&G loan: Permit No. 358-1787) in Point Hope, Alaska. Heart subsampling included approximately 10-20g of the full thickness from each region, including the left and right ventricles, left and right atria, and the interventricular septum (IVS). Kidney subsamples included approximately 4g each of cortex and medulla. A 10g subsample of skeletal muscle and liver were also collected. All tissues were kept partially frozen during subsampling. Tissues were sliced using stainless steel disposable knives (1 knife per region), placed in 2oz polyethylene Nasco™ whirl paks and stored at -20F prior to freeze drying. Freeze drying of samples was performed using a Freezone 4.5 Freeze Dry System (Labconco, Kansas City, MO). Percent moisture of tissues was calculated: [(wet weight - dry weight) / wet weight] X 100. Freeze dried tissues were homogenized prior to analysis.

1.3.2 Mercury analysis

Approximately 0.010g (liver and kidney) and 0.020g (skeletal muscle and heart) of homogenized powdered tissues were analyzed for [THg] using a DMA-80 direct mercury analyzer (Milestone, Inc, Shelton, CT; EPA Method 7473) [19]. All samples were analyzed in triplicate and measurements were considered acceptable at $\leq$ 10% error from the mean. Each run included one blank, a liquid standard (0.1µg/g $HgCl_2$ or 1µg/g $HgCl_2$ standard; Perkin Elmer, Waltham, Massachusetts) and two certified reference materials (DORM 3 = 0.382 ± 0.060µg/g and DOLT 4 = 2.58 ± 0.22µg/g; National Research Council Canada, Institute for National Measurement Standards, Ottawa, Canada). The detection limits were 0.075 µg/g (0.374 µM), for 0.010g of tissue, and 0.038µg/g (0.189

µM), for 0.020g of tissue. Recovery range of standard and certified reference materials were 93-100% (0.1µg/g $HgCl_2$ standard), 99-104% (1µg/g $HgCl_2$ standard), 96-111% (DORM 3) and 102-114% (DOLT 4). Reported data did not correct for the average recovery because observed values were considered within error of 100%.

1.3.3 Selenium analysis

Approximately 0.10g of cardiac muscle (left ventricle only) and 0.025g of kidney (cortex and medulla) were digested by microwave using nitric acid (HNO_3) and hydrogen peroxide (H_2O_2). An aliquot of this digest was reduced (Se^{VI} to Se^{IV}) in the presence of hydrochloric acid (HCl) at 95^o C for 90 minutes [19]. The digests were analyzed by a flow injection atomic spectroscopy-mercury/hydride system (Perkin Elmer, FIAS-MHS and AAnalyst 800). All samples were analyzed in triplicate and measurements were considered acceptable with $\leq$ 10% error from the mean. For each set of digestions, quality control samples included a blank, spike (1µg/g SeO_3^{2-} standard; Perkin Elmer, Waltham, Massachusetts), duplicate, sample spike and certified reference material (DOLT 4 = 8.30 ± 1.3µg/g; National Research Council Canada, Institute for National Measurement Standards, Ottawa, Canada). The detection limits were 0.20µg/g (2.50µM) for 0.10g of tissue, and 0.79µg/g (9.99µM) for 0.025g of tissue. Recovery range of quality control samples and certified reference materials were 74-83% (1.10µg/g blank spike), 72-99% (sample spike), and 71-91% (DOLT 4). Reported data did not correct for the average recovery because observed values were considered within error of 100%.

1.3.4 Statistical analysis

Due to limited samples sizes for ringed and spotted seals, only bearded seal data were used for statistical analyses. Samples below Hg detection level (heart: n = 4; kidney medulla: n = 2) were included in the summary statistics

(Table1.1) (removed for additional analysis) by assigning them a value that was half of the THg detection limit for that specific tissue. To determine whether [THg], [TSe], and Se:Hg molar ratios varied with sex and age for bearded seal tissues, separate ANCOVAs were assessed using sex as a factor and age as a covariate. Logarithmic transformations were used to meet ANCOVA assumptions.

Significant differences in [THg] within cardiac tissue (by cardiac region) of bearded seals were assessed using repeated measures ANOVA to account for matched samples. A 1-way ANOVA was conducted using logarithmic transformation of [THg] in liver, kidney cortex, kidney medulla, skeletal muscle and heart to determine difference among each tissue. Significant difference in [TSe] among kidney cortex, kidney medulla, and heart were assessed using a 1-way ANOVA. Separate pairwise t-tests with Bonferroni corrections were used to determine significant differences between [TSe] and [THg] in kidney cortex, kidney medulla, and heart. After logarithmic transformation of Se:Hg molar ratios, significant differences in Se:Hg molar ratios among kidney cortex, kidney medulla and heart were assessed using a 1-way ANOVA. If significant differences were found, each 1-way ANOVA was followed by a Tukey multiple comparisons test to determine significant differences among the tissues types. Rank order of [THg], [TSe] and Se:Hg molar ratios was determined after applying a rank transformation and conducting an ANOVA on those ranks to determine greater than (>) or less than (<).

A MANOVA was used to test the null hypothesis that all Se:Hg molar ratios are equal to 1 (unity) against the alternative hypothesis that all ratios are different from 1. The MANOVA was followed by a Hotellings T^2 to determine whether the Se:Hg molar ratio was different from 1 for all tissues. Statistical analysis was conducted using R version 2.15.3 [20]. Differences were considered significant at $p < 0.05$.

1.4 Results

Mean [THg] (Figure1.1) distribution in the heart was determined to be significantly different among the five cardiac regions evaluated ($p < 0.05$). The heart left ventricle [THg] was used to compare heart with [THg] of other tissues and other statistical assessments. Due to limited sample numbers for ringed and spotted seals, effect of sex or age could not be assessed. For bearded seal heart left ventricle [THg] (sex: $F = 0.07$, $p > 0.05$ / age: $F = 9.50$, $p < 0.05$) and Se:Hg molar ratios (sex: $F = 0.09$, $p > 0.05$ / age: $F = 5.28$, $p < 0.05$) varied significantly with age regardless of sex; [TSe] (sex: $F = 0.28$, $p > 0.05$ / age: $F = 1.14$, $p > 0.05$) did not. Bearded seal kidney medulla [THg] (sex: $F = 0.86$, $p > 0.05$ / age: $F = 0.15$, $p > 0.05$), [TSe] (sex: $F = 0.70$, $p > 0.05$ / age: $F = 0.04$, $p > 0.05$) and Se:Hg molar ratios (sex: $F = 0.09$, $p > 0.05$ / age: $F = 0.15$, $p > 0.05$) did not vary significantly with sex and age. Bearded seal kidney cortex [THg] (sex: $F = 0.02$, $p > 0.05$ / age: $F = 32.61$, $p < 0.05$) and Se:Hg molar ratios (sex: $F = 0.93$, $p > 0.05$ / age: $F = 28.73$, $p < 0.05$) varied significantly with age regardless of sex; [TSe] (sex: $F = 2.00$, $p > 0.05$ / age: $F = 0.05$, $p > 0.05$) did not. Bearded seal skeletal muscle [THg] (sex: $F = 0.17$, $p > 0.05$ / age: $F = 0.32$, $p > 0.05$) did not vary significantly with sex and age. Bearded seal liver [THg] (sex: $F = 0.06$, $p > 0.05$ / age: $F = 15.92$, $p < 0.05$) varied significantly with age regardless of sex.

Mean [THg] (Table 1.1) rank order among various tissues of bearded seals was: liver > kidney cortex > kidney medulla > skeletal muscle > heart ($p < 0.001$). Mean [THg] in bearded seal liver was 6 times greater than kidney cortex and over 100 times greater than heart left ventricle. Mean [THg] in bearded seal kidney was 2 times greater in cortex than medulla. Mean [THg] in bearded seal skeletal muscle was 3 times greater than in heart left ventricle.

Mean molar-based [THg] and [TSe] (Figure 1.2) were ranked as follows: kidney cortex > kidney medulla > heart left ventricle and [TSe] was greater than [THg] in all three tissues ($p < 0.001$). Mean [TSe] in kidney cortex and kidney

medulla of bearded seals were 4 and 6 fold greater than heart left ventricle, respectively. Mean Se:Hg molar ratios (Figure 1.3) in heart left ventricle and kidney medulla were significantly different and were greater than kidney cortex ($p < 0.001$). All Se:Hg molar ratios were greater than 1 ($p < 0.001$).

1.5 Discussion

1.5.1 Heart and kidney

Age was determined to be an important variable to consider in Hg and Se studies with respect to interpretation as well as study design [1,17]. In the heart and kidney, Hg has been implicated with hypertension and heart disease [11,12,21,22]. We examined [THg] distribution among and within regions of bearded seals heart and kidney and compared these to liver and skeletal muscle as well. Mean [THg] in the heart regions studied were determined to be significantly different. Due to low [THg] in the heart regions of bearded seals, these relatively small differences are not likely toxicologically significant. However, when sampling to determine cardiac [THg], consistency in selecting a heart region for sampling may be important (e.g., sample only left ventricle).

The distribution of [THg] in the kidney of bearded seals was similar to that of terrestrial and laboratory animals with a greater [THg] in the cortex than in the medulla [22,23]. Transport of Hg to the kidney is mediated by sulfur-containing compounds in the blood such as albumin, GSH, and cysteine. However, accumulation of Hg in the kidney is largely due to the presence of GSH in the proximal tubules. The proximal tubules are a major component of the nephron located in the kidney cortex. Thus care must be taken when sampling kidney and we encourage standardized protocols related to the relative amount of renal cortex and medulla used (e.g., select cortex or medulla or if both are to be used keep the proportion consistent).

Younger seals tend to have smaller renicules making it difficult to distinguish and discretely dissect cortex and medulla and therefore both regions

could be homogenized together in a standardized manner (e.g., intact renicules). Selecting a specific age cohort for monitoring purposes is an option as adult seals have relatively large kidneys and specific subsampling of kidney cortex is fairly easy. Thus [THg] variation with age and the differences by renal cortex and medulla can be easily accounted for. However, sampling the adult kidney for Hg biomonitoring should include known proportions of cortex and medulla in order to compare [THg] in kidney of young and older animals if desired. When evaluating Hg concentrations in the kidney, it is important to note the most abundant form is likely inorganic mercury (Hg^{2+}) [17,22,24]. $MeHg^{+}$ is demethylated to Hg^{2+} within the kidney and liver where it can be excreted into bile and urine or sequestered.

1.5.2 Traditionally sampled tissues

Mean [THg] in liver and skeletal muscle of bearded seals were similar to those found in previous studies [16–18]. In some marine mammals, liver is known to accumulate more Hg than other tissues mainly due to a detoxification function [1,3]. Approximately 90% of THg in the adult seal liver is in the form of Hg^{2+} and mostly bound to selenoproteins [1,17]. One study determined that approximately 53% of Hg in the adult seal liver is bound to Se and therefore biologically inactive [1].

Skeletal muscle of bearded seals had a greater [THg] than heart (cardiac muscle) which is consistent with the observation that skeletal muscle in diving mammals tend to have higher myoglobin concentrations than cardiac muscle [25,26]. Myoglobin plays a major role in storing oxygen and facilitating oxygen diffusion within muscle cells [27]. Redistribution of blood flow toward the heart and brain and away from other organs is a diving adaptation to maintaining an adequate blood supply to the heart during long periods of hypoxia thus reducing the need for increased myoglobin concentrations in cardiac muscle [8]. As myoglobin includes the sulfur-containing amino acid cysteine, it may provide a

target for $MeHg^+$ binding. Some studies have determined that approximately 80% or more of THg in skeletal muscle is in the form of $MeHg^+$ [3,17] and does not vary with age, making this tissue ideal for monitoring $MeHg^+$ exposure since age may be less of a confounder.

1.5.3 Se:Hg molar ratios

Differences in [TSe] and [THg] between the kidney medulla and heart may contribute to the observed differences in mean Se:Hg molar ratios within these tissues. Mean Se:Hg in heart and kidney medulla were greater than kidney cortex potentially due to a greater amount of [THg] in the kidney cortex. In the human kidney cortex under conditions of low Hg concentration, the Se:Hg molar ratio was as high as 300 (minimum [THg] = 0.005 µg/g, [28]) which is greater than what was found in kidney cortex of bearded seals (minimum [THg] = 0.164 µg/g) in the current study. In previous studies, Se:Hg ratios of 1 in the kidney were indicative of the formation of insoluble Hg-Se compounds found in marine mammals [5,29]. However, we determined that all Se:Hg molar ratios in bearded seal heart, kidney cortex, and kidney medulla, were greater than 1 indicating adequate supply of Se under additional oxidative stressors such as Hg.

1.5.4 Se as an antioxidant

Zenteno et al. [9] determined that production of superoxide($O_2^{\bullet -}$) was higher in the heart, kidney and muscle of ringed seals than in non-diving mammals such as pigs. Despite this, no increase in lipid peroxidation was reported in tissues of seals when compared to tissues of pigs indicating the presence of an effective and active antioxidant system in diving mammals [8,9]. Several studies have used GP_x activity as an indicator of antioxidant defenses along with superoxide dismutase, catalase and glutathione-S-transferase [8,30,31]. However, of the Se enzymes only GP_x synthesis and activity is dependent on Se [32,33].

The activity of GP_x was found to be higher in the heart, kidney and muscle of diving mammals than non-diving mammals with no difference in activity between deep-diving and shallow-diving mammals [8,31]. One can hypothesize that this diving adaptation may also be important in reducing damage caused by various forms of Hg that are known to cause oxidative stress. It is also possible that greater [TSe] in the kidney than in other tissues, as found in the current study, is a potential indication of an enhanced defense mechanisms which is consistent with greater production of $O_2^{\bullet -}$ in the kidney after ischemia/reperfusion episodes [8,9,34].

1.6 Conclusion

In bearded seals, like in many other marine mammals, liver has the highest concentration of Hg followed by kidney and skeletal muscle. Hg in the heart of bearded seals varied by heart region and therefore future studies should use consistent sampling methods in order to determine and compare Hg concentrations. The kidney cortex is the main accumulation site for Hg within the kidney of bearded seal despite the differences in seal kidney structure when compared to terrestrial mammals and requires consideration in future sampling designs. Se:Hg molar ratios greater than 1 in all tissues can be considered a baseline for normal Se concentrations under relatively low Hg concentrations. Greater [TSe] in the kidney are potentially indicative of greater antioxidant defense due to the redistribution of blood to the heart and greater production of reactive oxygen species in the kidney due to ischemia/reperfusion episodes during diving as well as a response to Hg.

1.7 Acknowledgements

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1.8 Figures

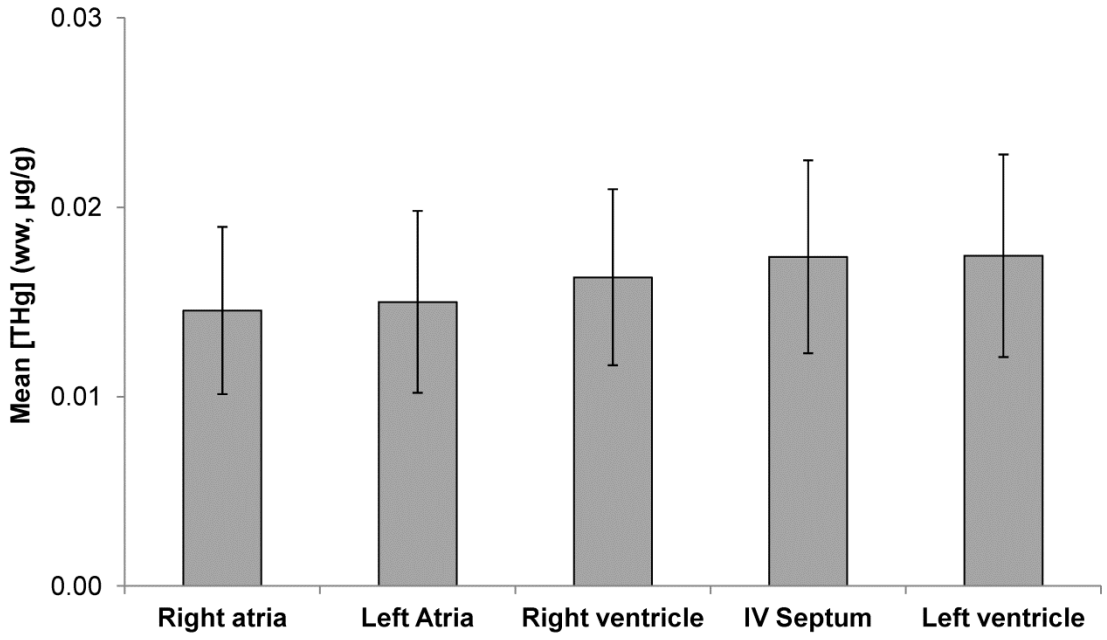

Figure 1.1: Total Hg concentrations in heart regions of bearded seals. Mean (± standard deviation) concentration of mercury in 5 regions of the heart. [THg] were ranked as follows: Right atria = Left atria < Right ventricle < Interventricular (IV) septum = Left ventricle ($p < 0.001$).

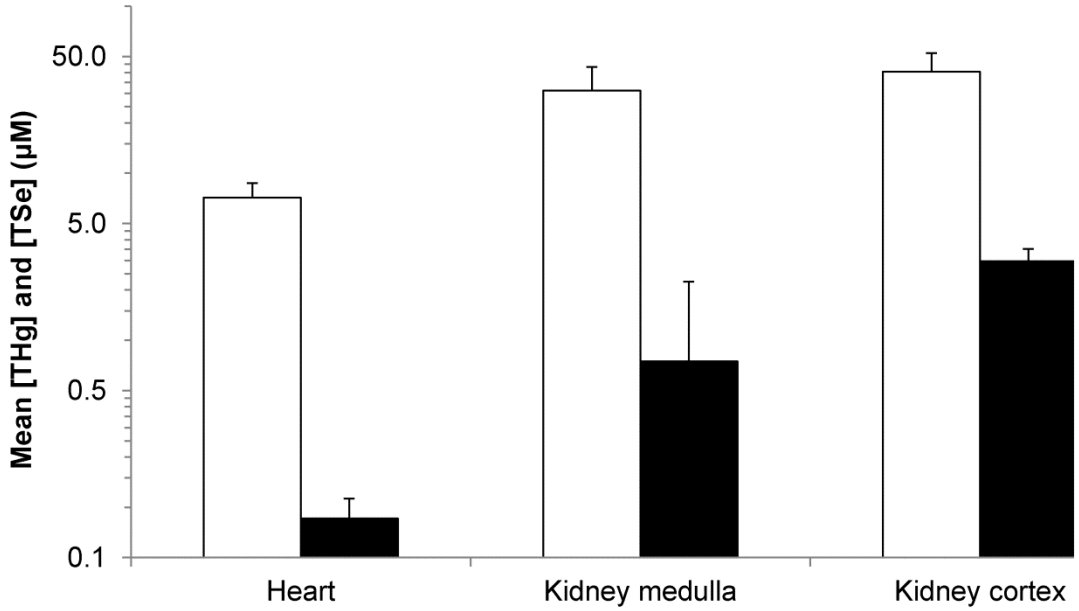

Figure 1.2: Molar concentrations of Hg and Se in bearded seals. Mean (+ standard deviation) molar concentration of mercury (black) and selenium (white) in heart (left ventricle), kidney cortex and kidney medulla of bearded seals. [THg] and [TSe] were ranked as follows: kidney cortex > kidney medulla > heart ($p < 0.001$). [TSe] > [THg] in all three tissues ($p < 0.001$).

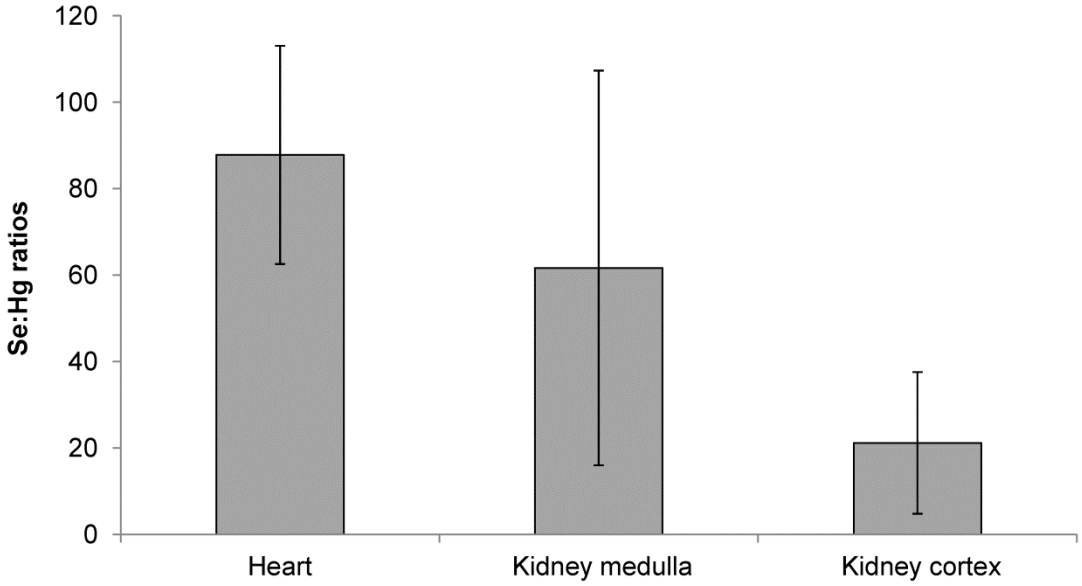

Figure 1.3: Se:Hg molar ratios in tissues of bearded seals. Mean (± standard deviation) Se:Hg molar ratio in heart (left ventricle), kidney cortex and kidney medulla tissue of bearded seals. Statistical differences among tissues were: heart > kidney medulla > kidney cortex ($p < 0.001$). All ratios were greater than 1 ($p < 0.001$).

1.9 Tables

Table 1.1: Total Hg and Se concentrations in tissues of ice seals. Mean, standard deviation (SD), and median for total Hg and Se concentrations in tissues of ice seals[a]

	Bearded		Ringed		Spotted	
	THg	*TSe*	*THg*	*TSe*	*THg*	*TSe*
Heart						
Mean	0.02	0.61	0.09	0.44	0.16	0.38
SD	0.01	0.44	0.06	0.10		
Median	0.02	0.56	0.09	0.38		
n	42	42	7	7	1	1
Kidney cortex						
Mean	0.55	3.15	0.54	1.83	0.83	2.59
SD	0.32	0.96	0.30	0.41		
Median	0.47	2.98	0.65	1.79		
n	41	41	7	7	1	1
Kidney medulla						
Mean	0.32	2.43	0.23	1.22	0.38	1.38
SD	0.11	0.94	0.08	0.30		
Median	0.13	2.27	0.24	1.29		
n	41	41	7	7	1	1
Skeletal muscle						
Mean	0.05	ND	0.14	ND	0.26	ND
SD	0.03		0.08			
Median	0.04		0.14			
n	42		7		1	
Liver						
Mean	3.06	ND	2.35	ND	1.14	ND
SD	2.92		1.83			
Median	2.16		2.91			
n	43		7		1	

[a] µg/g wet weight; age from <1 to 30 years old
- THg = total mercury; TSe = total selenium
- ND = not determined

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CHAPTER 2:

Assessment of mercury tissular and body burden and selenium concentrations in 5 Steller sea lion pups from the Aleutian Islands[1]

2.1 Abstract

Total mercury ([THg]) and total selenium ([TSe]) concentrations were measured in several tissue compartments to determine specific tissue burdens and body burden of THg in Steller sea lion (*Eumetopias jubatus)* pups. Mercury (Hg) tissue compartments and body burdens were calculated using fresh weight of each biological tissue (compartment mass) multiplied by the [THg] for each specific tissue (compartment). Hair [THg] among the 5 pups ranged from 1.78 – 59.17µg/g. Despite the apparent differences in THg exposure based on hair [THg], relative [THg] and THg burden (rank order and proportion of total amounts) for the tissue compartments were very similar. In all 5 pup tissue sets the highest [THg] determined was in hair and the lowest [THg] determined was in bone. In all 5 pups, pelt, muscle and liver compartment burdens were among the highest as percent of Hg body burden. The [THg] and THg burden determined in hair and muscle related well to other tissue Hg levels indicating utility for these commonly monitored tissues to estimate Hg status of other compartments. In 4 of 5 pups the Se:Hg molar ratios in all tissues ranged from 1.13 to 50.12. The pup with the highest hair [THg], as compared to other pups, had Se:Hg molar ratios in 9 of 14 tissues that were 0.7 or less potentially indicating an inadequate supply of Se for that particular pup.

Keywords: mercury, selenium, burden, molar ratio, Steller sea lions

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2.2 Introduction

Mercury (Hg) is a naturally occurring element with known toxic effects in humans and terrestrial mammals. Recently, there has been increasing concern over total Hg concentrations ([THg]) found in some Steller sea lions (*Eumetopias jubatus)* in the western Aleutian Islands [1]. Steller sea lions are piscivorous marine mammals that biomagnify relatively high Hg concentrations through the diet and easily transfer Hg through the placenta to the developing fetus similar to other piscivorous mammals [2,3]. Steller sea lions from the Aleutian Islands are part of the western distinct population segments (DPS) which are genetically different from the Steller sea lions found in Southeast Alaska. The Steller sea lion populations in the western DPS, particularly those in the western Aleutian Islands, have been slow to recover from dramatic population declines that occurred during the 1970s and 1980s [4–7]. Several causes have been hypothesized, such as nutritional stress, fisheries competition, and chemical pollution among others, but no studies have produced conclusive evidence [4,8–10]. Chemical pollution, including exposure to contaminants such as polychlorinated biphenyls (PCB) and Hg, has been under study for several years as a potential cause for the lack of recovery of Steller sea lions in the western DPS [1,8,11,12]. Determining Hg distribution in Steller sea lion tissues (and in piscivorous marine mammal tissues in general) is an important step in understanding Hg toxicity in this species, especially in the fetus and neonate as the cohort of concern.

Toxicity of Hg is dependent on the bioavailability and chemical form of Hg which dictates distribution among tissues. Monomethyl mercury ($MeHg^+$) has been known to have adverse effects on reproductive, immunological, and neurological functions in humans and rats [13–16]. $MeHg^+$ can cross the blood-brain barrier as well as other membrane structures such as the placenta and gastrointestinal tract, and > 90% of ingested $MeHg^+$ can be absorbed into blood [2,3,17]. Thus, $MeHg^+$ distribution is systemic, reaching all vital organs including

the brain, and accumulates in several tissues including erythrocytes, muscle, and hair [16]. On the other hand, inorganic mercury (Hg^{2+}) is generally found in greater concentrations in two target organs, the liver and kidney. Several studies have found that both liver and kidney have demethylating mechanisms that convert $MeHg^{+}$ to Hg^{2+} [16,18,19]. Demethylating mechanisms develop with increasing age leaving very young mammals vulnerable to $MeHg^{+}$ toxic effects.

Hair has been commonly used as an indicator tissue for $MeHg^{+}$ exposure. Hair total Hg concentration ([THg]) is highly correlated with [THg] in blood and is thought to be a good indicator of THg in circulation [1,11,20]. Keratin is the main structural protein found in hair as well as nails and skin and consists of multiple disulfide cross linkages as well as cysteine residues. Cysteine is a sulfur-containing amino acid and is likely the binding site for Hg in hair. It is estimated that approximately 80% of total mercury (THg) in hair is in the form of $MeHg^{+}$ [21]. In recent studies [THg] in hair of Steller sea lion pups have been found to be higher in the Aleutian Islands when compared to Southeast Alaska [1,11,12] and in some cases have exceeded the human and wildlife threshold for Hg adverse effects [22,23]. Steller sea lion pups are born with a natal pelage (lanugo) that is molted when they are 4 to 6 months old. Therefore, Hg in the hair of pups under 4 months of age represents Hg exposure via placental transfer from the mother during gestation. Since it is more difficult to sample tissues such as muscle, liver, kidney and brain in live pups, this study will determine how representative hair [THg] is of other tissue compartments.

Some have hypothesized that Hg toxicosis in humans and marine mammals can be diminished through the association of selenium (Se) and Hg particularly when the Se:Hg molar ratio is greater than 1 in the kidney, liver and possibly other tissues [24–27]. The antioxidant Se is an essential trace element in the mammalian diet under homeostatic control not only due to its importance in antioxidant defense but also likely due to its direct interactions with toxic elements such as Hg. These interactions play a role in the tissue distribution of

[THg] and total selenium concentrations ([TSe]) and thus are important for interpretation of total Hg body burden. Selenium can increase Hg half-life in the blood and liver, make it less reactive, and have a significant effect in organ distribution and excretion of Hg [27,28]. Selenium tends to be higher in marine mammals when compared to terrestrial mammals likely due to a greater intake of Se in the marine diet. High Se intake is also an advantage for marine mammals in that it can play a physiological role as an antioxidant for diving mammals as well as a role in ameliorating adverse effect of Hg.

Evaluating THg body burden along with individual tissue compartment burdens and concentrations in pinnipeds such as Steller sea lion pups will provide understanding about Hg distribution and storage in various biological tissues. In particular, this will put concentrations of THg measured in traditionally sampled tissues (e.g., hair, liver and skeletal muscle) into better perspective (e.g., % of total body burden). We compare [THg] from all tissues collected from pup carcasses, taking into account mass of tissue, to determine rank order of tissues from the highest to lowest [THg] and THg burden. We evaluate the [TSe] in order to determine Se:Hg molar ratio among tissues of the body to provide insight on the possible protective role of Se within the whole body as well as specific tissue compartments.

2.3 Materials and Methods

2.3.1 Sample collection

Steller sea lion pups (n = 5) found dead on their natal rookeries in the Aleutian Islands, Alaska in 2011 and 2012 were collected by the Alaska Department of Fish and Game (ADF&G) (MMPA/ESA Permit No. 14325). Necropsies of thawed animals were performed under an educational outreach setting with university student volunteers assisting in total body measurements (weight, length, girth, etc.), external examination, sex determination, tissue collection, and dissection under professional guidance and instruction. All

tissues were processed and analyzed whole except pelt, muscle and bone. All subsamples were collected using stainless steel disposable scalpels and cutting knives. A subsample of hair was removed from the pelt using battery operated grooming clippers (Wahl ® Super Pocket Pro ® Clippers). Hair samples were washed in 1% Triton X-100 following previously published hair washing protocol for metals analysis [1,11,20]. A 5g subsample of the pelt (hair, epidermis, and dermis) was collected to represent whole pelt [THg]. Muscle subsamples for THg analysis were collected in equal proportion by mass from the scapular region and the pelvic region of each pup. All remaining skeletal muscle was removed from bone and weighed. Total skeletal muscle mass was a combination of the scapular region subsample, pelvic region subsample, and skeletal muscle removed from bones.

One femur and a cartilaginous rib (4^{th} rib) were used to represent bone [29]. Cartilage was separated from bone and its weight was added to the final bone mass. Bone was placed in distilled water for 3 weeks to soften the remaining fascial tissue for removal. Bone was then air dried under a fume hood and dry weight was recorded. Freeze drying of samples was performed using a Freezone 4.5 Freeze Dry System (Labconco, Kansas City, MO). Percent moisture of most tissues was calculated: [(wet weight - dry weight) / wet weight] X 100. Homogenization of complete tissue compartments and subsampled tissues was performed using a Retsch Cryomill (Retsch Inc, Newton, PA). All samples were stored in polyethylene Whirlpaks ® and 1 gallon Ziploc ® bags at -20ºF and -80ºF prior to freeze drying. Dry homogenized samples were stored short-term at room temperature and returned to ADF&G for archiving after chemical analysis.

2.3.2 Mercury analysis

Approximately 0.010-0.020g of homogenized powdered tissues were analyzed for [THg] using the Direct Mercury Analyzer (Milestone, Inc, Shelton,

CT; EPA Method 7473) [1,11,20,30]. Approximately 0.0060g of hair was analyzed separately from the total pelt. All samples were analyzed in triplicate and were considered acceptable with a 15% error from the mean. Each run included one blank, a liquid standard (0.001µg/g $HgCl_2$ or 1µg/g $HgCl_2$ standard; Perkin Elmer, Waltham, Massachusetts) and two certified reference materials (DORM 3 = 0.382µg/g and DOLT 4 = 2.58µg/g; National Research Council Canada, Institute for National Measurement Standards, Ottawa, Canada). The detection limits were 0.075 µg/g (0.374 µM) for 0.010g of tissue and 0.038µg/g (0.189 µM), for 0.020g of tissue. Recovery range of standard and certified reference materials were 87-101% (0.010µg/g $HgCl_2$ standard), 94-104% (1 µg/g $HgCl_2$ standard), 91-118% (DORM 3) and 102-114% (DOLT 4). Reported data did not correct for the average recovery because observed values were considered within error of 100%.

2.3.3 Selenium analysis

Approximately 0.030-0.050g of each homogenized powdered tissue was digested by microwave using nitric acid (HNO_3) and hydrogen peroxide (H_2O_2) and analyzed for selenium concentration following the methods in Chapter 1. For each set of digestions, quality control samples included a blank spike (Perkin Elmer, Waltham, Massachusetts), duplicate, sample spike, internal standard (DORM 3) and certified reference material (DOLT 4 = 8.30µg/g; National Research Council Canada, Institute for National Measurement Standards, Ottawa, Canada). The detection limit range was 0.39-0.66µg/g (4.99-8.32µM). Recovery range of quality control samples and certified reference materials were 80-111% (1.01 µg/g blank spike), 89-122% (sample spike), and 78-108% (DOLT 4). Reported data did not correct for the average recovery because observed values were considered within error of 100%.

2.3.4 Data analysis and calculations

Due to the small sample size (n = 5) of the data, significant differences were not assessed. A cumulative rank was applied to [THg] and THg tissue burden data. The cumulative rank was determined by assigning each tissue compartment with a value of 1 to 15 with 1 being the tissue with the highest [THg] or THg tissue burden and 15 being the tissue with the lowest [THg] or THg tissue burden. Each pup had a different set of values by tissue type depending on the [THg] distribution and THg tissue burden. The sum of these values for each tissue for the 5 pups was determined to be the cumulative rank number for that specific tissue (i.e., brain THg cumulative rank: 6 + 13 + 7 + 11 + 14 = 51). Spearman correlation (r_s) was used to assess the association between [THg] and THg tissue burdens for the group of pups.

Total Hg tissue compartment burdens (mg) were calculated as the product of tissue mean [THg] (mg/g, wet weight) and wet tissue mass (g). Total Hg body burden for each pup was calculated as the sum of all Hg tissue compartment burdens. Molar concentrations (μM) of THg and TSe were calculated as the product of THg and TSe mass based concentrations (μg/g) and the molecular weight of each element (Hg = 200.59μg/mole; Se = 78.96μg/mole).

Percentages (%) of body mass, of [THg], and of THg body burden were calculated as follows: % body mass = (Mass of tissue compartment / weight of pup) X 100; % [THg] = ([THg] of tissue compartment / sum of [THg] for all tissue compartments) X 100; and % THg body burden = (THg tissue compartment burden / THg body burden) X 100.

2.4 Results

Necropsy results could not determine a cause of death; 2 of the 5 pups were emaciated with no evident subcutaneous blubber upon gross examination. Concentrations of THg in hair samples (a tissue traditionally sampled from live captured animals) varied more than 30-fold among the 5 pups (Table 2.1). For

all 5 pups the highest [THg] for a tissue was in hair and the lowest [THg] was in bone (Table 2.2A). In 4 of the 5 pups the highest percent of THg burden was in pelt and in all 5 pups the lowest percent of THg burden was in spleen (Table 2.2B). Table 2.3 summarizes [THg], THg burden, [TSe] and Se:Hg molar ratios. A comparison of [THg], tissue mass, and THg burden demonstrated the significance of individual tissue mass to the overall burden of THg in the body (Figure 2.2). Together pelt, muscle and liver comprised 86% of total Hg body burden. Of this, pelt and muscle comprised 65% of total body mass, 22% of the sum of [THg] in the body, and 73% of THg body burden. THg burden for various tissue compartments was highly correlated with [THg] in some tissues (r_s = 0.90 or greater, Figure 2.3A, B). THg burden and [THg] in various tissues were both highly correlated with THg burden in muscle and [THg] in muscle, respectively (r_s = 0.90 or greater, Figure 2.4A, B). Hair [THg] was correlated with target organ [THg] (r_s = 0.60 or greater, Figure 2.5A) including brain, heart and kidney. Hair [THg] was also correlated with target organ THg tissue burdens (r_s = 0.60 or greater, Figure 2.5B).

Mean Se:Hg molar ratios demonstrated a high variability among tissues. Bone had the highest mean Se:Hg molar ratio and hair had the lowest mean Se:Hg molar ratio (Figure 2.6). In 4 of 5 pups the Se:Hg molar ratios in all tissues ranged from 1.13 to 50.12. In the pup with the highest hair [THg] (pup no. 5), the Se:Hg molar ratios in 9 of 14 tissues were 0.7 or less.

2.5 Discussion

2.5.1 Total Hg concentrations

Although [THg] in the tissues of these carcasses cannot be considered representative of the western DPS Steller sea lion population, they do span the range of hair [THg] found in live captured pups in this population and provide a unique opportunity to study how THg is distributed among different tissue compartments of sea lion pups based on both mass (mg of THg) and

concentration (µg/g, ww of THg). THg concentrations in hair have been used as an indicator of Hg exposure in Steller sea lion pups due to ease of collection in live capture studies [10–12] and has been shown to correlate closely to circulating blood [THg] concentrations in young pups [11].

In the current study, 2 of the 5 pups were equal to or exceeded the threshold for [THg] in hair set by the EPA illustrating recent concerns. Similar to the findings of Brookens et al. [29] in harbor seals (*Phoca vitulina*), hair was found to have the highest [THg] and bone was found to have the lowest [THg] for all 5 pups. Hair has been known to be an excretory pathway for Hg [20,29]. While bone may serve as a reservoir for trace metals such as lead, previous studies have shown that Hg is not retained in bone to the same extent as other metals [29].

Sea lions in the current study are < 1-2 months of age and likely have not developed significant demethylation mechanisms. Therefore it is likely that a high proportion of the THg in the liver and kidney of the 5 pups is $MeHg^{+}$. Liver and kidney [THg] have not been extensively studied in sea lion pups. Holmes et al. [8] is the only published study to date that evaluated [THg] in various tissues of Steller sea lion young of the year from both the Aleutian Islands and Southeast Alaska. In the current study 4 of 5 pups were males and had higher [THg] in brain, heart and lungs when compared to the Aleutian Island males from Holmes et al. [8]. These same 4 pups had lower [THg] in kidney and liver but similar concentrations in testes to those found in the Aleutian Island males. These differences may be age dependent as the Aleutian Island males were approximately 1 year old from Holmes et al. [31] and the males in the current study were < 1-2 months old. Mercury is known to bioaccumulate in liver and to some extent in kidney resulting in higher [THg] with increased age.

Mercury concentrations in blood, hair and muscle can provide information regarding Hg exposure but are not considered key target organs. Mercury concentrations in various tissues can be most useful when mass of the tissue

(compartment) is taken into account so as to determine the actual burden. Total Hg body burdens were determined by the sum of all tissue compartment burdens. The ranking of total Hg burden of tissues in all 5 pups was similar to that found in Pacific harbor seal pups. The tissue compartments with the highest % THg body burden were pelt, muscle and liver and the lowest % total Hg body burden was in bone. The combined % total Hg body burden in pelt and muscle for sea lion pups was slightly lower than what was found in Pacific harbor seal pups (greater than 75%) [29]. The conceptual diagram designed after [29] illustrates how THg is distributed in the body and where the highest THg burden is found (Figure 2.7).

Hair [THg] was correlated with [THg] and with THg tissue burdens in target tissues such as the brain, heart, and kidney, indicating that hair [THg] is a good indicator of THg exposure and Hg tissue burden in pups of this age. The close association between muscle [THg] and both tissue [THg] and tissue THg burden of other tissue compartments also indicated that muscle was an adequate tissue for determining THg exposure and THg tissue burden.

2.5.2 Se:Hg molar ratios

Previous studies on marine mammals indicate a strong correlation between Se and Hg concentrations [24,25]. It is hypothesized that an abundance of Se as compared to Hg on a molar basis, where Se:Hg molar ratio is well above 1, is important for potential amelioration of the adverse effects due to $MeHg^{+}$ exposure and maintenance of Se-dependent processes [24,25,27,32]. One study showed that Se antagonism of Hg only occurred after Hg threshold concentrations had been reached [27]. In the current study, only 2 of 5 pups appear to have reached the Hg threshold designated for humans under acute Hg exposure. In 4 of 5 pups, Se:Hg molar ratios were equal to or higher than 1.0 in all tissues. The pup with the highest hair [THg] had ratios at 0.7 or less in liver,

brain, heart, and spleen, among others indicating a potentially inadequate Se supply for normal function and protection against Hg toxicosis.

2.5.3 Se concentrations

Sea lions are piscivorous diving mammals and tend to have higher Se concentrations than non-diving mammals through their marine diet. This is an advantage for a marine mammal because Se is a major component of Se-dependent glutathione peroxidase and other enzymes which help control the over production of reactive oxygen species caused by episodes of ischemia (restriction of blood flow) and reperfusion (return of blood flow) that occur in diving mammals [33,34]. Most Steller sea lion pups are thought to be weaned at approximately 1 year of age and while they are capable of entering the water and swimming soon after birth, they do not engage in diving for foraging purposes. Se in the liver and kidney of young of the year animals (< 1 year old) is thought to be an early store of Se from placental transfer to the fetus, this along with Se in milk are the only sources of Se available until the young animals can utilize their own Se sources through foraging. Some studies in humans and dairy cattle indicate that milk is a good source of Se for young mammals and it is representative of Se obtained through maternal diet [35,36].

Selenium tissue concentrations have not been previously published for Steller sea lion pups. Se concentrations in the liver of 3 of 5 pups (mean: 2.35 $\underline{+}$ 1.25) were slightly higher than what was found for Pacific harbor seal pups (mean: 0.75 + 0.05) [37]. Despite the high variability in tissue [THg] among the 5 pups, Se concentrations were within a narrow range particularly in brain, heart, and skeletal muscle. Brain, liver and erythrocytes can synthesize selenoproteins from Se reservoirs in those tissues, independent of the Se found in the serum and obtained through the diet [38]. Heart, kidney and skeletal muscle as well as other tissues rely solely on the Se content in serum as well as the diet indicating a greater dependence on dietary Se for normal function.

2.6 Conclusion

Hair had the highest [THg] in all 5 Steller sea lion pups as compared to other tissue compartments. Since these pups were only 1-2 months of age, the hair (lanugo) sampled was a good indicator of Hg exposure via maternal placental transfer and potentially a good indicator of individual Hg tissue burdens. Bone had the lowest [THg] and is not a significant reservoir for Hg. The percent of total Hg body burden for many organs in Steller sea lion pups was similar to that found in Pacific harbor seals [29]. The Se:Hg molar ratios were between 1 and 50 in all tissues of 4 of the 5 pups. The pup with the highest [THg] in all tissues had Se:Hg molar ratios of 0.7 or less in 9 of 14 tissues (including brain, heart, liver and spleen) indicating that this animal may have limited Se-dependent protection.

2. 7 Acknowledgements

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2.8 Figures

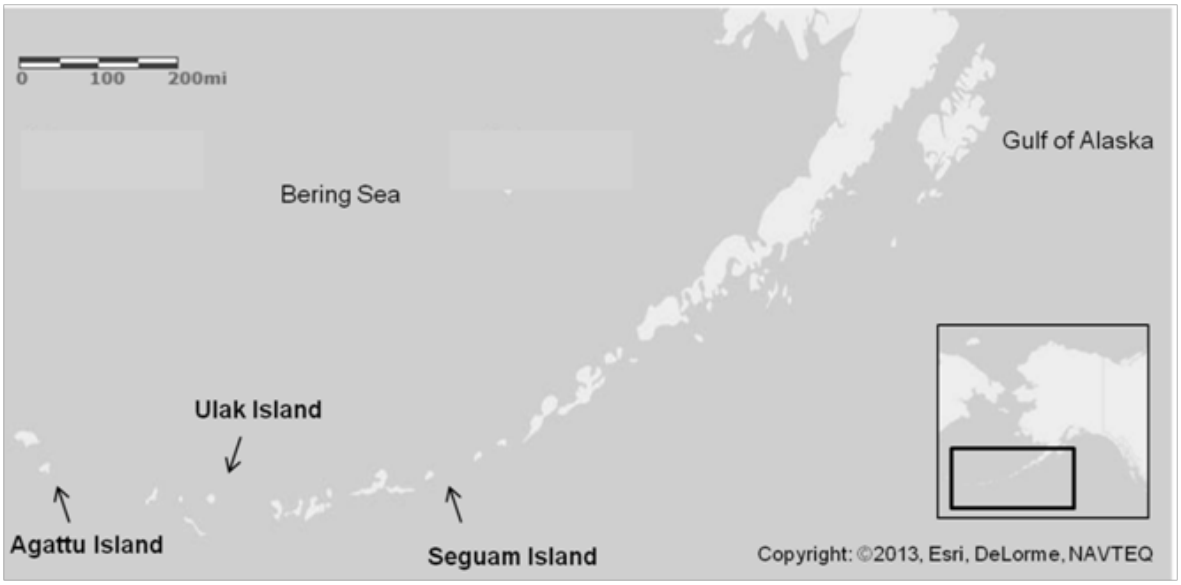

Figure 2.1: Steller sea lion pup collection site map. Collection sites of dead Steller sea lion pups from the Aleutian Islands include Agattu Island (52°26’07”N, 173°34’32”E), Ulak Island (51°21’54”N, 178°56’50”W) and Seguam Island (52°19’24”N, 172°27’58”W). The arrow tip points to the sample location.

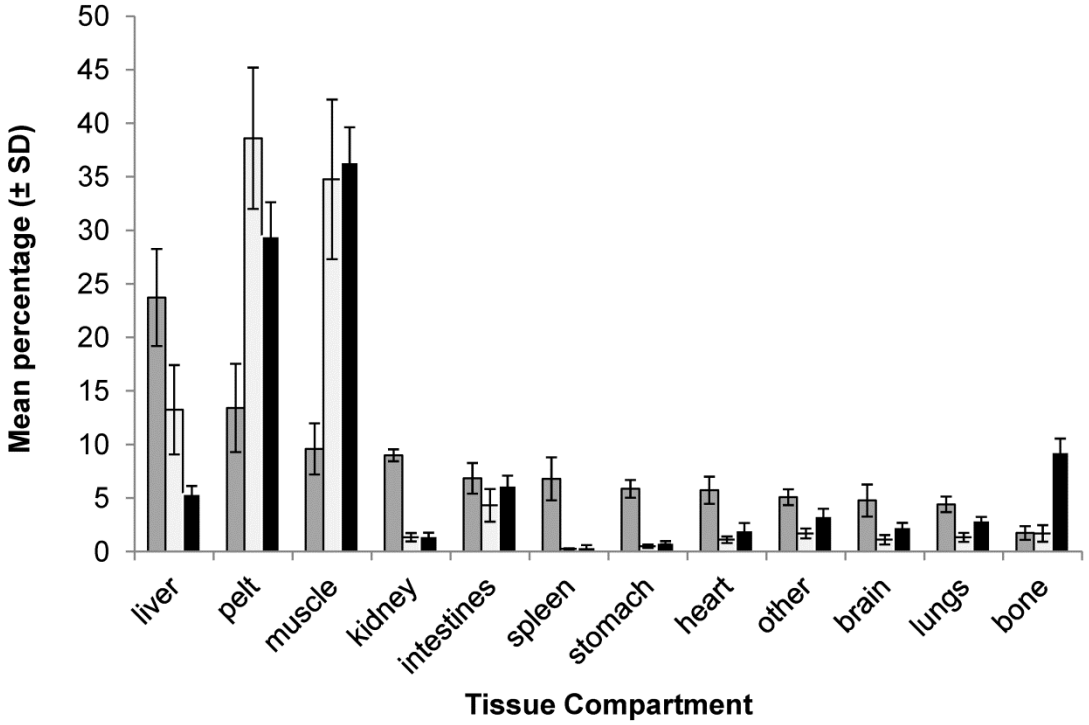

Figure 2.2: Mean % of [THg], THg tissue burden, and tissue mass. Mean (± standard deviation) percentage of sum of total mercury (THg) concentration (µg/g; grey), THg body burden (mg; white), and tissue mass (g; black) for tissues of 5 Steller sea lion pups. Pelt was comprised of hair, dermis, and epidermis. "Other" was comprised of tongue, esophagus, diaphragm. Intestines were comprised of pancreas, small and large intestine.

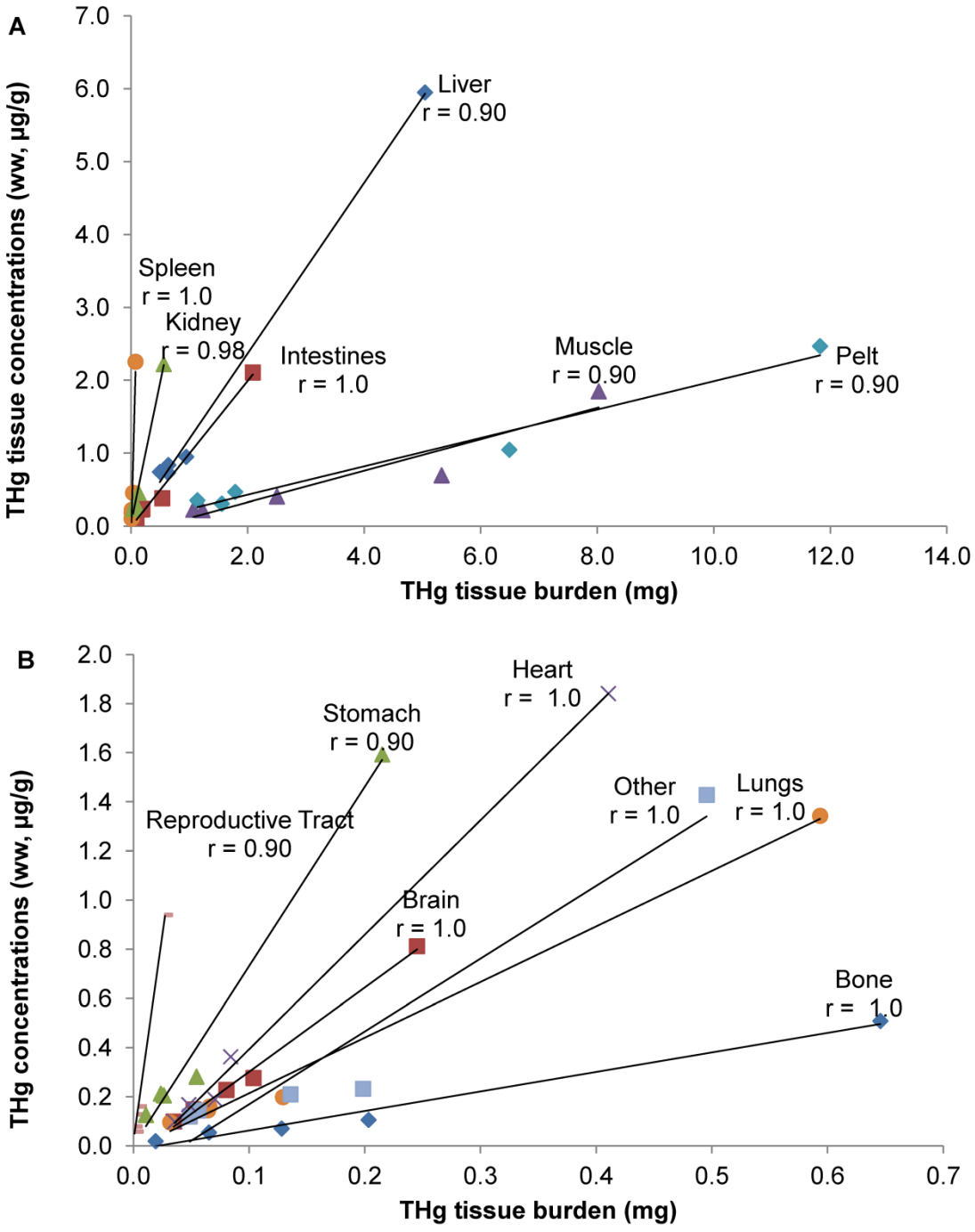

Figure 2.3: High [THg] (A) and Low [THg] (B) vs. THg tissue burden. Total mercury (THg) tissue concentration (ww, µg/g) in relation to THg tissue burden (mg) for tissues of 5 Steller sea lion pups. Spearman correlations ($r = r_s$) were assessed using R programing.

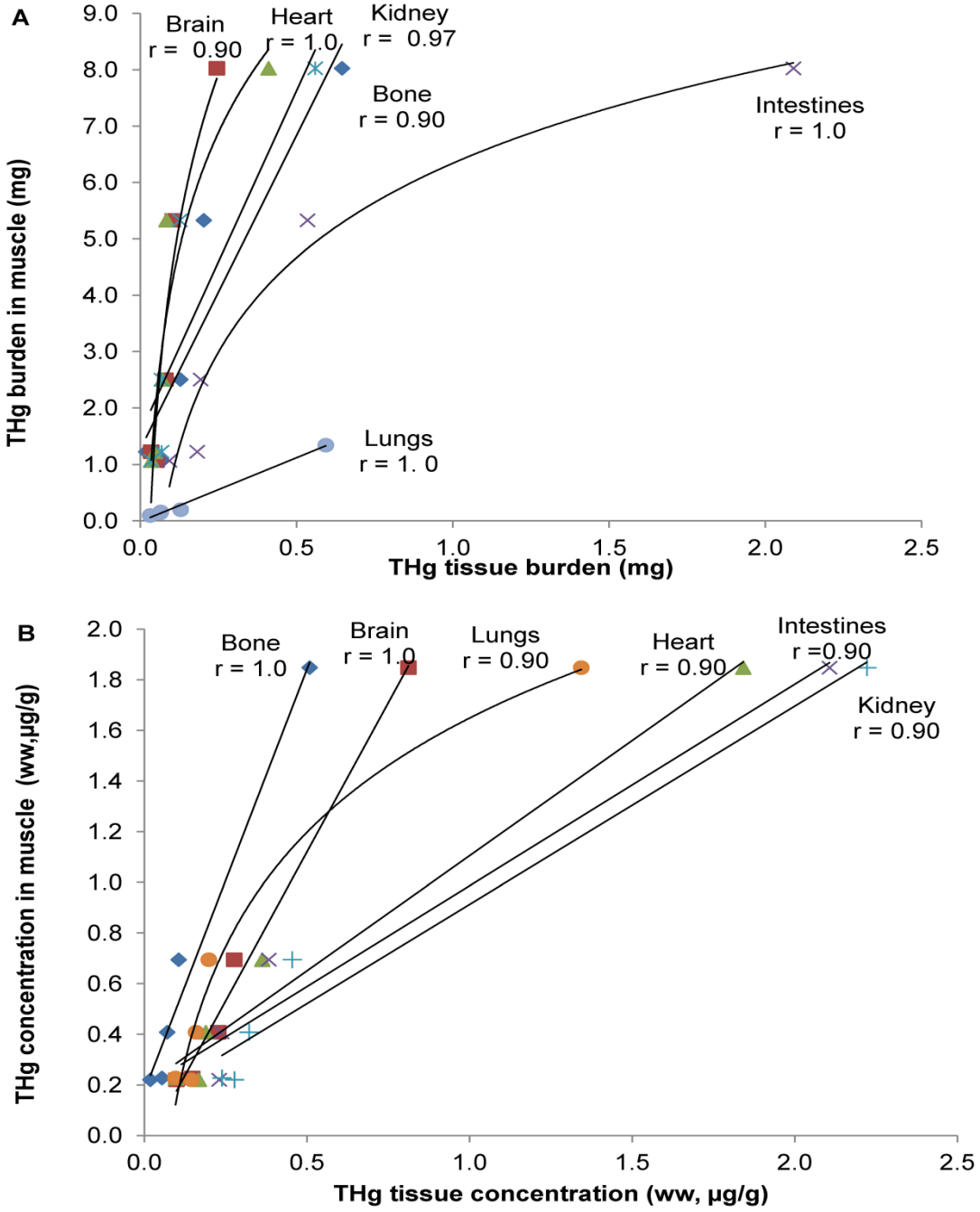

Figure 2.4: [THg] (A) and THg burden (B) of tissues vs. muscle [THg]. Total mercury (THg) tissue concentration (ww, µg/g) and total mercury tissue burden (mg) of non-muscle tissue compartments of 5 Steller sea lion pups in relation to muscle. Spearman correlations ($r = r_s$) were assessed using R programing.

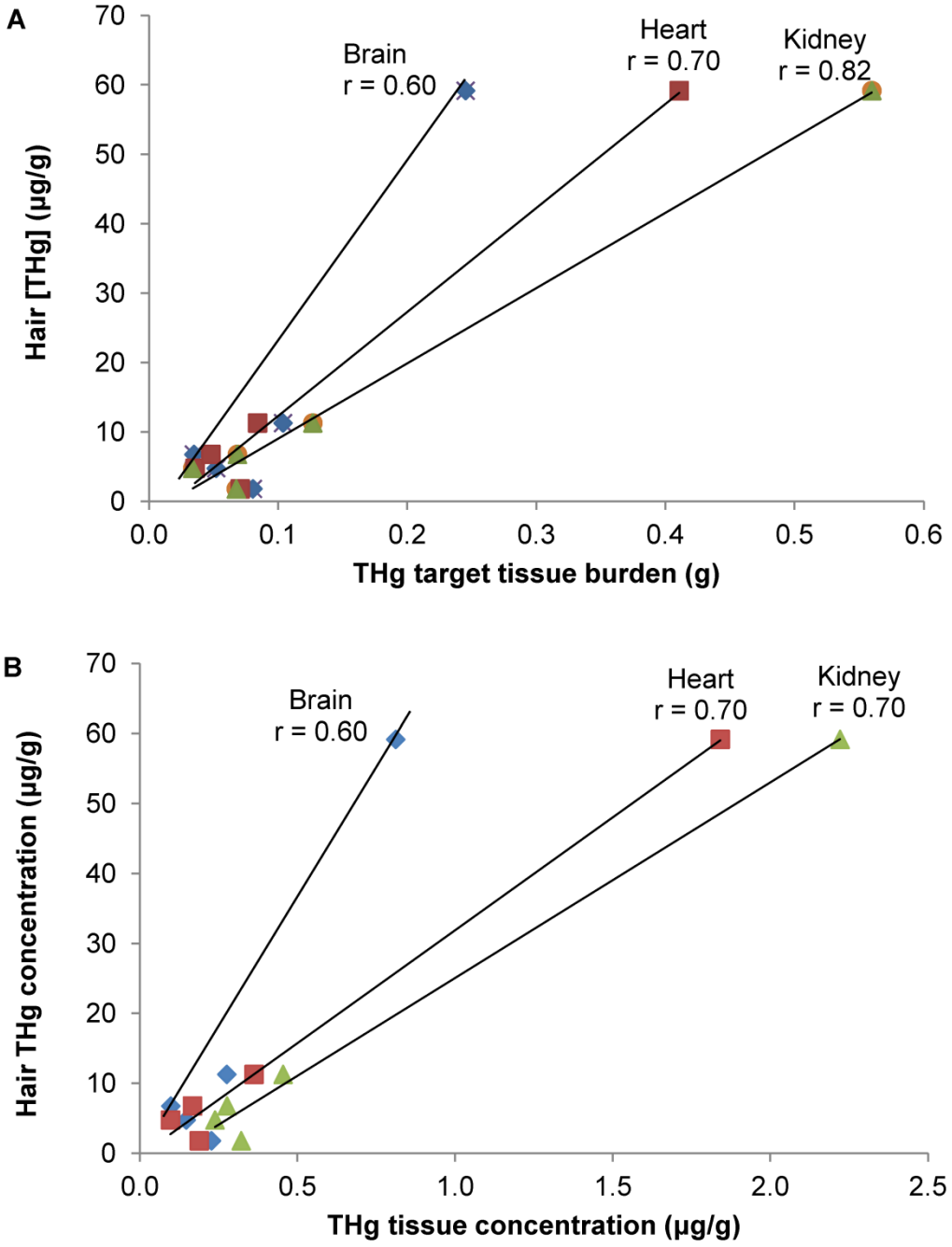

Figure 2.5: [THg] (A) and THg burden (B) of tissues vs. hair. Total mercury (THg) non-hair tissue concentration (ww, µg/g) and total mercury non-hair tissue burden (mg) in tissue compartments of 5 Steller sea lion pups in relation to total mercury concentrations in hair. Spearman correlations ($r = r_s$) were assessed using R programing.

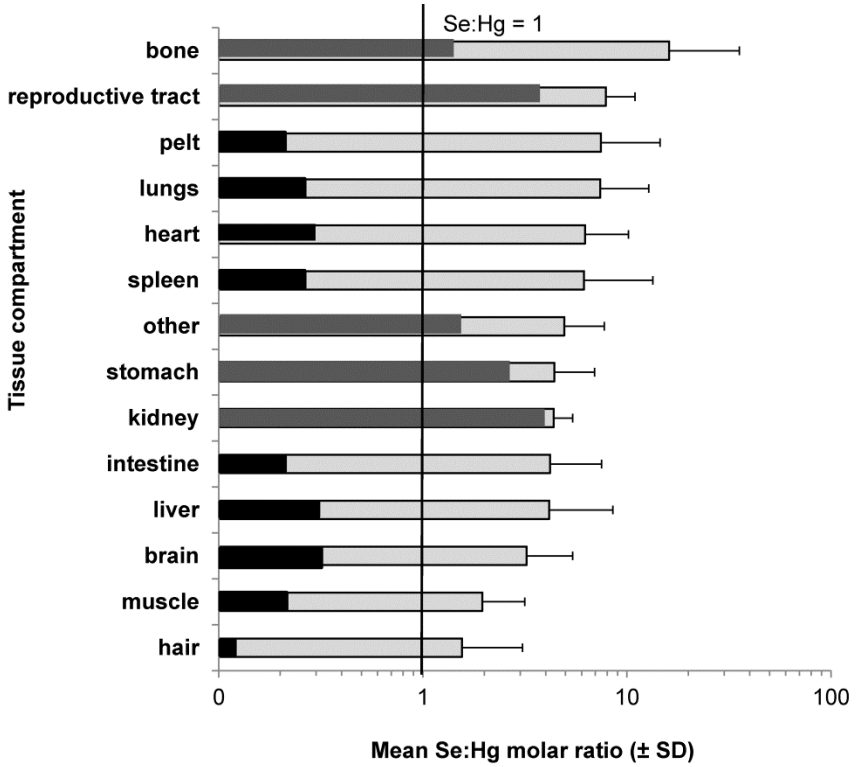

Figure 2.6: Se:Hg molar ratios in tissues of Steller sea lion pups. Mean (± standard deviation) Se:Hg molar ratio in various tissues of 5 Steller sea lion pups (light grey). Se:Hg molar ratios for the case study (dark grey; tissues in black are below 1) are included in the mean.

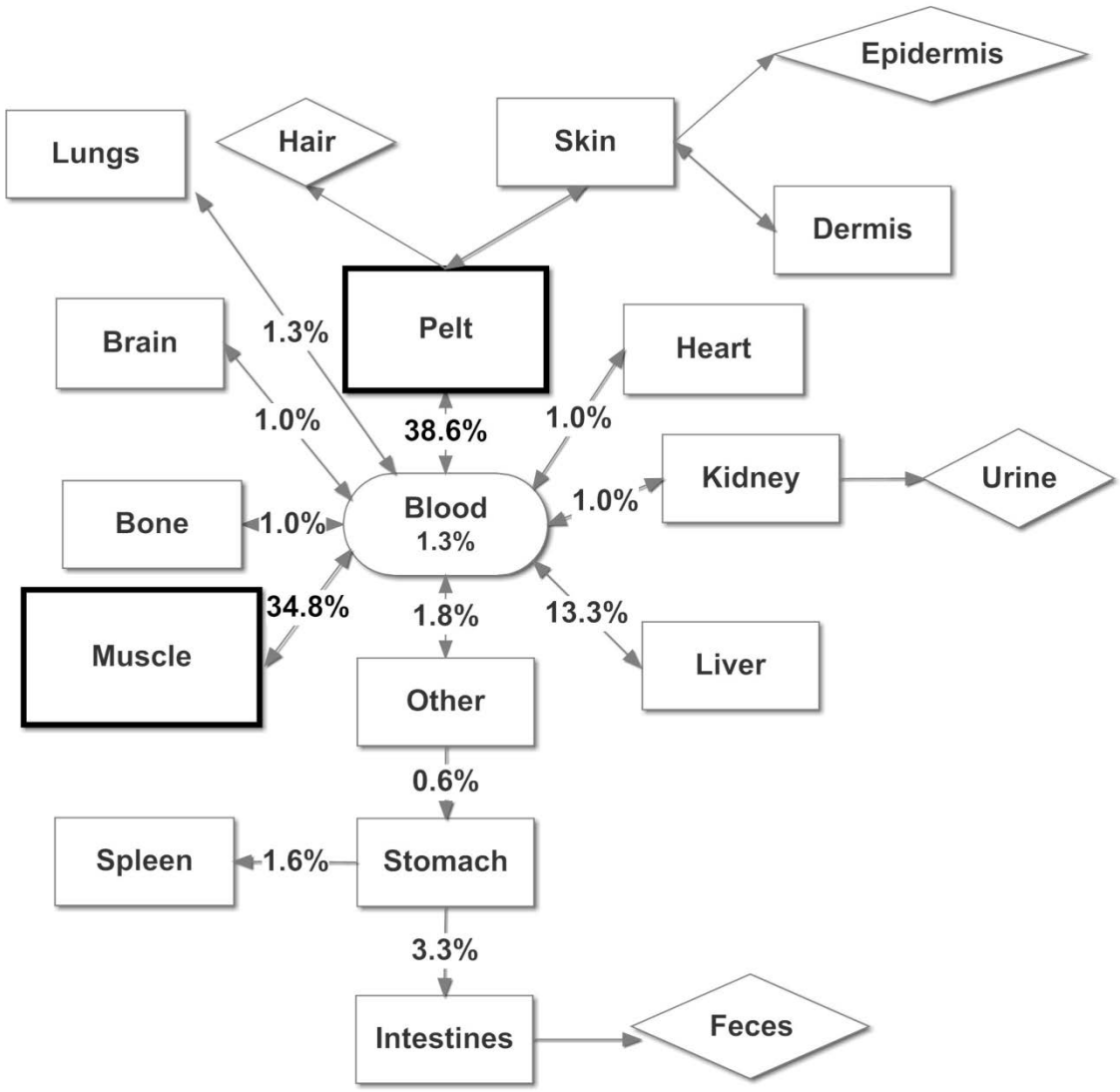

Figure 2.7: Conceptual model for mean % of THg body burden. Mean percent of THg body burden in various tissue compartments of Steller sea lion pups. All tissue compartments that exchange THg with blood are represented with rectangles; tissue compartments considered not to exchange THg with blood are represented with diamonds.

2.9 Tables

Table 2.1: Steller sea lion pup collection. Location (rookery site of collection), year collected, body weight, sex, total mercury concentration in hair and total mercury body burden (calculated from the sum of compartment burdens reported in Table 2.2) for 5 Steller sea lion (*Eumetopius jubatus*) pups.

Pup No.	Location	Year	Body Weight (kg)	Sex	Hair THg (µg/g)	THg body burden (mg)
1	Ulak Island	2012	14.6	M	1.78	3.08
2	Agattu Island	2011	19.1*	M	6.75	4.16
3	Ulak Island	2012	19.5	M	4.75	5.48
4	Seguam Island	2012	26.4	M	11.27	14.24
5	Agattu Island	2011	19.1*	F	59.17	30.25

-THg = total mercury
-*Pups were emaciated with no grossly visible subcutaneous blubber

Table 2.2: [THg] (A) and THg tissue burden (B) cumulative rank. Total mercury concentration (A) cumulative rank order [a,b,c] and total mercury burden (B) cumulative rank order for multiple tissues (compartments) from 5 Steller sea lion pups [a,b,c]

A

pup1	pup2	pup3	pup4	pup5
hair	hair	hair	hair	hair
liver	liver	liver	pelt	liver
pelt	pelt	muscle	liver	pelt
kidney	kidney	kidney	muscle	spleen
muscle	intestines	pelt	kidney	kidney
brain	spleen	intestines	spleen	intestines
epidermis	epidermis	brain	intestines	muscle
stomach	muscle	stomach	heart	heart
other	stomach	other	epidermis	stomach
intestines	heart	heart	stomach	epidermis
spleen	lungs	spleen	brain	other
heart	other	lungs	other	lungs
lungs	brain	epidermis	lungs	brain
bone	bone	bone	bone	bone

Tissue	Cumulative[c] Rank
hair	5
liver	11
pelt	16
kidney	22
muscle	27
heart	34
brain	38
stomach	44
spleen	47
other	48
intestines	51
epidermis	53
lungs	61
bone	70

[a] Pelt includes hair, dermis, epidermis

[b] Highest total mercury concentration = hair; lowest total mercury concentration = bone

[c] lowest possible score = 5; highest possible score = 70

B

pup1	pup2	pup3	pup4	pup5
pelt	pelt	muscle	pelt	pelt
muscle	muscle	pelt	muscle	muscle
liver	liver	liver	liver	liver
intestines	intestines	intestines	intestines	intestines
bone	kidney	other	bone	bone
brain	lungs	bone	other	lungs
other	other	brain	lungs	kidney
heart	heart	heart	kidney	other
kidney	brain	kidney	brain	heart
lungs	stomach	lungs	heart	brain
stomach	bone	stomach	stomach	stomach
spleen	spleen	spleen	spleen	spleen

Tissue	Cumulative[c] Rank
pelt	6
muscle	9
liver	15
intestines	20
bone	31
brain	38
heart	38
kidney	39
other	40
lungs	45
stomach	49
spleen	60

[a] Pelt includes hair, dermis, epidermis

[b] Highest mercury burden = pelt; lowest mercury burden = spleen

[c] lowest possible score = 5; highest possible score = 60

Table 2.3: [THg], THg tissue burden, [TSe] and Se:Hg ratios. Mean (± SD) and range of total mercury concentration, total mercury tissue burden, total selenium concentration and Se:Hg molar ratio in tissues of 5 Steller sea lion pups[a]

Tissue	THg	THg burden	TSe	Se:Hg
hair	16.75 ± 23.96	ND	3.31 ± 0.97	1.56 ± 1.53
	1.78 - 59.17		2.08 - 4.75	0.14 - 4.17
liver	1.84 ± 2.30	1.30 ± 1.96	1.15 ± 1.37	5.76 ± 4.38
	0.74 - 3.95	0.49 - 5.05	0.43 - 3.52	0.44 - 10.68
pelt	0.93 ± 0.91	3.04 ± 4.61	1.06 ± 0.81	9.25 ± 7.09
	0.31 - 2.47	1.14 - 11.82	0.40 - 2.11	0.48 - 17.42
kidney	0.70 ± 0.85	0.43 ± 0.22	1.57 ± 1.44	4.48 ± 1.04
	0.24 - 2.22	0.03 - 0.56	0.47 - 3.74	2.61 - 5.10
muscle	0.68 ± 0.68	2.40 ± 2.99	0.33 ± 0.05	3.51 ± 1.20
	0.22 - 1.85	1.07 - 8.03	0.25 - 0.38	0.52 - 3.51
spleen	0.64 ± 0.91	0.03 ± 0.03	0.39 ± 0.19	4.23 ± 7.24
	0.10 - 2.25	0.01 - 0.08	0.27 - 0.76	0.62 - 18.52
intestine	0.61 ± 0.84	0.53 ± 0.84	0.46 ± 0.11	3.71 ± 3.30
	0.11 - 2.11	0.09 - 2.09	0.22 - 0.49	0.51 - 8.74
heart	0.53 ± 0.74	0.23 ± 0.16	0.45 ± 0.07	5.36 ± 3.93
	0.10 - 1.84	0.04 - 0.41	0.41 - 0.59	0.69 - 10.69
stomach	0.48 ± 0.62	0.09 ± 0.09	0.93 ± 0.83	3.66 ± 2.54
	0.21 - 1.59	0.01 - 0.22	0.25 - 2.17	2.24 - 8.80
other	0.43 ± 0.56	1.66 ± 0.18	0.75 ± 0.58	4.34 ± 2.81
	0.12 - 1.43	0.05 - 0.50	0.25 -1.61	2.75 - 8.90
lungs	0.39 ± 0.54	1.42 ± 0.24	0.42 ± 0.20	4.94 ± 5.40
	0.10 - 1.34	0.03 - 0.59	0.37 - 0.78	0.60 - 12.76
brain	0.31 ± 0.29	0.10 ± 0.08	0.33 ± 0.02	4.83 ± 2.20
	0.10 - 0.81	0.04 - 0.25	0.21 - 0.26	0.66 - 6.34
reproductive tract	0.27 ± 0.37	0.02 ± 0.01	0.66 ± 0.53	8.51 ± 3.15
	0.06 - 0.94	0.00 - 0.03	0.21 - 1.52	4.12 - 11.91
bone	0.15 ± 0.20	0.21 ± 0.25	0.31 ± 0.09	16.11 ± 19.42
	0.02 - 0.51	0.02 - 0.65	0.18 - 0.39	1.83 - 50.12
THg Body Burden		11.45 ± 11.40		

[a] µg/g wet weight; mg
- THg = total mercury; TSe = total selenium
- ND = not determined

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GENERAL CONCLUSION

This research project used biological tissues of ice seals and sea lions in order to determine total mercury (THg) and total selenium (TSe) distribution between various tissues of marine mammals. The Se:Hg molar ratio was calculated for various tissues in order to better understand the potential defense mechanism against Hg toxicosis in marine mammals. In chapter 1, the main tissues of interest were heart and kidney of bearded seals in order to determine improved sampling methods for biomonitoring, histopathology and biochemistry (oxidative and antioxidant mechanisms). In addition, liver and muscle were used as reference tissues for THg since the concentrations for these tissues are well known in the literature. In chapter 2, the main focus was determining THg tissue burden from [THg] of various tissues in Steller sea lion pups and obtaining the overall Hg body burden in order to gain better perspective of [THg] in traditionally sampled tissues (e.g. hair and skeletal muscle).

Chapter 1 determined that Hg concentrations in the heart varied across the regions. Due to relatively low Hg concentrations in the heart, these differences were not considered toxicologically significant. The kidney, however, showed distinct Hg accumulation in the cortex over the medulla indicating that Hg is not evenly distributed between regions. This distribution has excretion and toxicological implications, as well as for biomonitoring. While liver and muscle are traditionally used for Hg biomonitoring in ice seals, they are not considered directly representative of the potential target organs of Hg toxicosis such as the brain, heart and kidney. In part, this is a result of varying capacities of organs to accumulate $MeHg^{+}$ and/or Hg^{2+} (e.g.,demethylated $MeHg^{+}$). The Se:Hg molar ratios in the heart, kidney cortex, and kidney medulla of bearded seals were greater than 1 indicating an abundance of Se within these tissues and the potential for antioxidant –based protection.

Chapter 2 determined that like muscle, hair is well correlated with tissue THg concentrations and tissue THg burdens indicating that in cases where muscle samples are not available, hair can provide sufficient information of Hg exposure and burden within the body for numerous tissue compartments of Steller sea lion pups. The THg concentrations of specific tissues do not illustrate the full extent of Hg distribution and potential toxicosis. Tissue THg concentrations are more useful when mass of tissue is taken into account in order to determine tissue Hg burden that can then be summed among all compartments to estimate total body burden. The Se:Hg molar ratios in various tissues of sea lion pups were between 1-50 and appeared to decrease with increasing Hg concentration and burden but requires careful consideration due to our low sample number and one extreme case of high Hg levels. The pup with the highest THg concentration in hair (59.17 ug/g) showed a lower Se:Hg molar ratio in tissues such as brain and heart among others indicating an inadequate supply of Se for normal function in those target organs.